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13. ABSTRACT (Maximum 200 Words)

We previously demonstrated that the breast tumor kinase BRK regulates the RNA-binding protein Sam68 that plays roles in RNA metabolism. Two novel Sam68-like mammalian proteins SLM1 and SLM2 were identified, and the purpose of our study was to determine the roles of these RNA-binding proteins in breast cancer signaling. Cotransfection of normal murine mammary gland (NMuMG) cells with different BRK expression constructs and GFP-SLM1 or GFP-SLM2 revealed a direct correlation between BRK activity and SLM1 and SLM2 phosphorylation. Mutation of a regulatory terminal tyrosine in BRK (Y-F) increased the level of SLM protein phosphorylation. Phosphotyrosine immunoreactivity was enhanced in the nuclei of NMuMG cells when active BRK was expressed. Fractionation of cells transfected with BRK expression constructs showed that BRK(Y-F) was highly enriched in the nuclear fraction. In functional studies in NMuMG cells, we determined that BRK mediated phosphorylation of Sam68, SLM1 and SLM2 led to dramatic inhibition of their RNA-binding activities. In expression studies, only Sam68 was strongly coexpressed with BRK in normal and malignant breast epithelial cells, and it appears to be the major identified BRK nuclear substrate in these cells. Induction of BRK in breast tumor cells may lead to inappropriate phosphorylation and inhibition of Sam68.

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INTRODUCTION

The intracellular breast tumor kinase BRK/Sik was cloned from a human metastatic breast tumor in a screen for protein tyrosine kinases involved in breast cancer [1]. BRK/Sik is expressed in many breast carcinoma cell lines and in a high percentage of primary breast tumors that have been examined, but it has not been detected in normal human breast tissue [1, 2], or at any stage of mammary gland differentiation in the mouse [3]. Recent genetic studies indicate that high levels of BRK/Sik expression correlate with the postmenopausal status of breast cancer patients [4]. BRK/Sik was also cloned from cultured human melanocytes and designated PTK6 [5]. It was subsequently found to be expressed in metastatic melanoma cell lines but not in normal melanocytes [6]. We identified the mouse orthologue of BRK/Sik (Sik for Src-related intestinal kinase), in the mouse small intestine [7, 8]. BRK/Sik is expressed throughout the human gastrointestinal tract and moderate increases in BRK/Sik levels were detected in colon tumors [3]. Our recent data indicate that BRK/Sik is expressed in nuclei of normal prostate epithelial cells in men and mice, but it is relocalized to the cytoplasm in PIN and in prostate tumors [4].

BRK/Sik belongs to a novel subfamily of intracellular tyrosine kinases that is distinct from the Src-family and includes Srm, FRK(Rak/Gtk/Iyk/Bsk) and Src42A/Dsrc41 (reviewed in [9]. These kinases share a low degree of sequence homology with known kinases, including one another, but the exon structure of the genes encoding these kinases is highly conserved and distinct. Lack of myristoylation and tissue specific expression distinguishes most BRK/Sik family kinases from the Src-family, and these proteins are not specifically targeted to the membrane. In fact, both BRK/Sik [10, 11] and FRK [12] have been shown to have nuclear substrates. The gene encoding Srm was cloned and disrupted in the early 1990's and lack of a prominent phenotype led to an apparent discontinuation of research on this protein [13]. Interestingly, growth inhibitory functions have been revealed for human FRK [12, 14] and drosophila Src42A [15]. Our preliminary data in the BRK/Sik knockout mouse model also suggest growth inhibitory roles for BRK/Sik in the gastrointestinal tract .

Substrates for BRK/Sik, include the novel adaptor-like protein BKS [16] and the nuclear RNA-binding protein Sam68 [10]. Sam68 is a member of the STAR family of KH-domain containing RNA-binding proteins that regulate RNA metabolism [17]. In some cell lines Sam68 is localized to unique nuclear structures termed Sam68/SLM nuclear bodies (SNBs) [18]. After

stress such as heat shock, Sam68 can also be found in stressed induced nuclear bodies [19]. Although Sam68 can be phosphorylated by other intracellular tyrosine kinases, only BRK/Sik has been shown to colocalize with Sam68 in the nucleus [10]. In normal prostate epithelial cells, BRK/Sik colocalizes with Sam68, but this colocalization is lost in PIN and in prostate tumors [4]. We recently identified additional substrates of the BRK/Sik kinase, the STAR family proteins SLM1 and SLM2 [20], that are a major focus of the studies described below.

Several studies indicate that Sam68 regulates RNA metabolism. Sam68 was found to act as a functional homologue of the human immunodeficiency virus type 1 (HIV1) Rev protein, which transports RNA from the nucleus to the cytoplasm [21, 22]. We showed that BRK/Sik phosphorylation of Sam68 inhibits its ability to bind RNA and to function as a cellular Rev homologue [10]. Sam68 has been shown to influence the utilization of specific RNAs in the cytoplasm and BRK/Sik also functionally regulates this activity [23]. In addition, Sam68 co-localizes with and associates with RNA splicing factors [19, 24], and was recently shown to be a regulator of alternative splicing [25].

While localized in the nucleus, BRK/Sik can phosphorylate and inhibit Sam68 RNA-binding. The ability of BRK/Sik to negatively regulate the RNA-binding activities of Sam68 may have an impact on cell growth and differentiation. Several studies suggest that RNA-binding functions of Sam68 regulate cell growth because a variant of Sam68 lacking a functional KH domain inhibits cell cycle progression [26]. Sam68 is also a direct target of Cdc2 during mitosis [27] and an extracellular signal-regulated kinase (ERK) target [25]. In the current study we investigated the significance of phosphorylation of the BRK/Sik nuclear substrates Sam68 and the novel related proteins SLM1 and SLM2 in mammary gland epithelial cells.

BODY

RESULTS SINCE LAST REPORT

Inhibition of SLM1 and SLM2 RNA Binding Activities

Two Sam68-like mammalian proteins, SLM1 and SLM2, were identified based on their high degree of sequence similarity with Sam68 within the GSG domains [20, 28]. We determined that both SLM1 and SLM2 are substrates of BRK/Sik (revised manuscript in preparation). SLM-1 shares many similarities with Sam68; it interacts with many of the same proteins and is also tyrosine phosphorylated by Src during mitosis [20]. SLM2 was also identified by its ability to interact with RNA-binding motif (RBM) in spermatogenesis and named T-STAR or ETOILE [29]. It can regulate the selection of alternative splice sites [30]. Because of the similarities that SLM1 and SLM2 share with Sam68, we asked if SLM1 and SLM2 are also BRK/Sik substrates.

In previous progress reports we demonstrated that SLM1 and SLM2 are novel substrates of the BRK/Sik kinase. To determine if BRK/Sik regulates RNA binding functions of SLM-1 and SLM-2, we performed RNA binding studies in the presence and absence of BRK/Sik. Normal murine mammary gland (NMuMG) cells were cotransfected with Myc-Sam68, Myc-SLM1 or Myc-SLM2 and the empty expression vector pcDNA3 or constitutively activated BRK/Sik Y-F. Total cell lysates were immunoblotted with anti-phosphotyrosine, anti-Myc and anti-BRK/Sik antibodies. The Myc-tagged constructs as well as BRK/Sik Y-F were expressed and increases in Myc-Sam68, Myc-SLM1 and Myc-SLM-2 tyrosine phosphorylation were observed (Figure 1). Sam68 served as a positive control, as we have shown previously that BRK/Sik can negatively regulate Sam68 homopolymeric RNA binding [10] (Figure 1). Cell lysates were divided equally and incubated with either poly(A), (G) or (U) immobilized to agarose or Sepharose alone. SLM-1 bound poly(A) and SLM-2 bound poly(G) homopolymeric RNA, when coexpressed with the empty expression vector. However, little or no RNA binding was detected when SLM-1 or SLM-2 were coexpressed with BRK/Sik Y-F (Figure 1). These data indicate that BRK/Sik negatively regulates the RNA binding abilities of SLM-1 and SLM-2.

Expression of BRK/Sik and STAR Proteins in different cell lines

A panel of different human tumor cell lines derived from various tissues, including breast, was screened for expression of BRK/Sik and the STAR protein family members Sam68, SLM-1 and SLM-2. BRK/Sik expression was detected in cell lines derived from intestine, breast,

prostate and oral epithelia (Figure 2), which represents the expression pattern previously shown [1-3, 11]. While Sam68 is ubiquitously expressed in all the cell lines, SLM-1 and SLM-2 show a more restricted expression pattern. Highest protein levels of SLM-1 were detected in the oral epithelial cell lines, where BRK/Sik is also expressed at relatively high levels. These data suggest that there is some tissue specificity in BRK/Sik regulation of STAR proteins, and that Sam68 and SLM-1 may be physiological relevant substrates of BRK/Sik in some cell types. Sam68 is strongly coexpressed with BRK/Sik in normal mammary gland epithelial cells and in breast cancer cell lines.

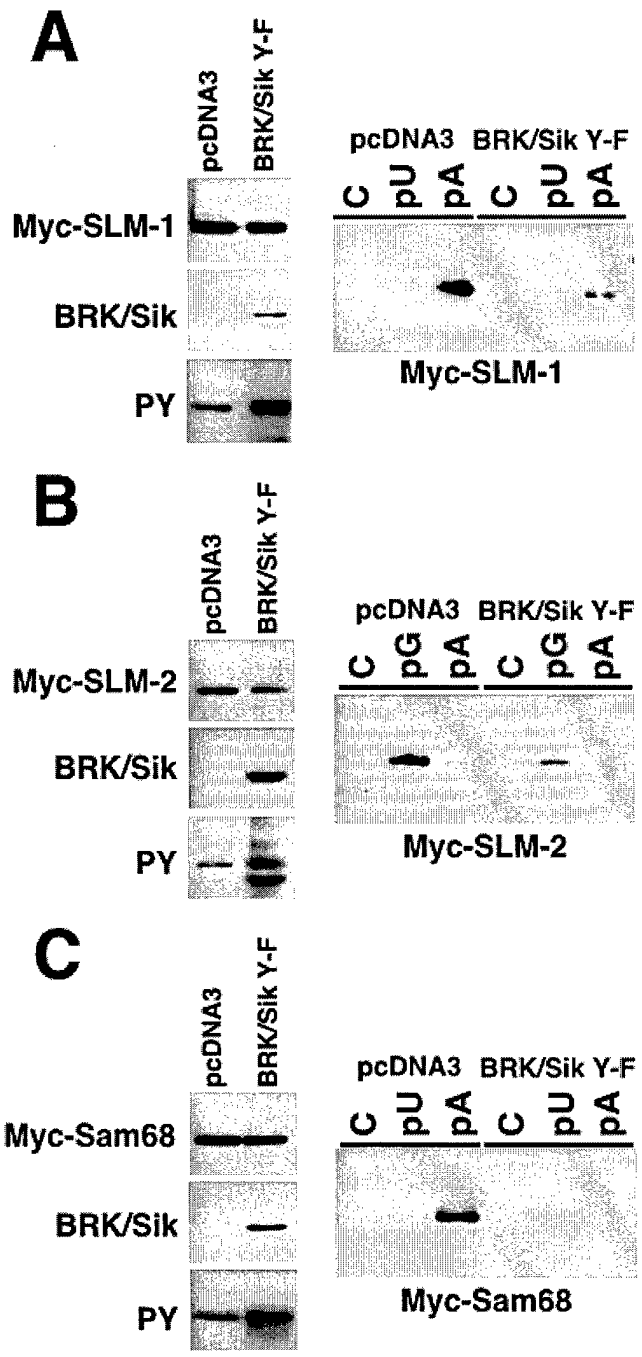

Figure 1. BRK/Sik negatively regulates the ability of SLM-1 and SLM-2 to bind RNA. NMuMG cells were cotransfected with Myc-epitope tagged SLM-1 **(A)**, SLM-2 **(B)** or Sam68 **(C)** and the expression vector pcDNA3 alone or activated BRK/Sik Y-F. Total cell lysates were divided equally and precipitated with Sepharose 4B as a control (C) or poly(U)-agarose (pU), poly(A)-agarose (pA) and poly(G)-agarose (pG), followed by anti-Myc immunoblotting (right panels). Aliquots of total cell lysates were probed for expression of Myc-SLM-1, SLM-2 or Sam68 and BRK/Sik Y-F as well as tyrosine phosphorylation (PY) of SLM-1, SLM-2 or Sam68 (left panels). The ability of Myc-SLM-1 to bind poly(A) agarose and Myc-SLM-2 to bind poly(G) agarose was inhibited in cells transfected with BRK/Sik Y-F.

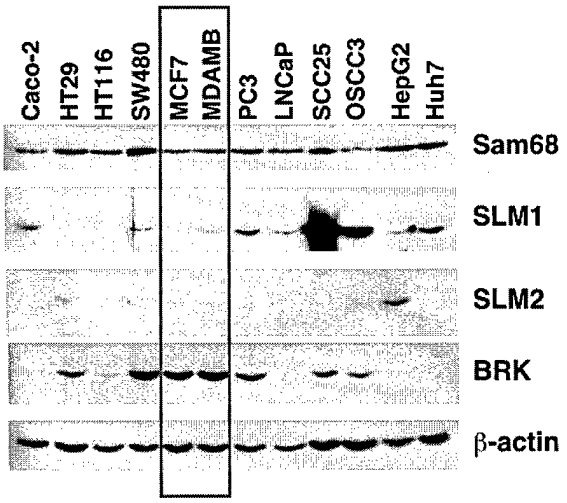

Figure 2. BRK/Sik and STAR protein expression in human epithelial cell lines. Total cell lysates were prepared from various epithelial cell lines and immunoblotted for the STAR proteins Sam68, SLM-1 and SLM-2 as well as for BRK/Sik. β-actin serves as a loading control. Sam68 is ubiquitously expressed in all of the cell lines screened, whereas SLM-1 and SLM-2 are only expressed in a subset of cell lines.

Expression of the BRK/Sik is Not Maintained in Normal Murine Mammary Gland Cells.

In transient transfection assays and in stable cell lines with constitutive expression of introduced constructs, we observed that expression of BRK/Sik is not maintained in normal murine mammary gland cells. As an example, total cell lysates from normal murine mammary gland (NMuMG) cells were prepared 24 to 72 h post transfection and immunoblotting was performed with antibodies specific for BRK/Sik, α-tubulin, and phosphotyrosine to determine the levels of protein expression and phosphorylation (Figure 3). As expected, cells transfected with wild-type BRK/Sik and BRK/Sik with a mutation of its carboxy terminal tyrosine Y447 to F (BRK/Sik Y-F) show an increased level of tyrosine-phosphorylated proteins. Unexpectedly, expression of transfected protein decreased as time went on and was highest at 24 hours post transfection. Interestingly, expression of the BRK/Sik KM construct that lacked kinase activity was most rapidly lost after transfection, indicating that some functions of BRK/Sik may be kinase independent. Rapid reduction in expression of transfected BRK/Sik suggests that cells expressing high levels of BRK/Sik may be lost through growth arrest or cell death. In addition, it raised the possibility that BRK/Sik expression may also be regulated at the posttranscriptional level.

To examine stability of the different BRK/Sik isoforms in NMuMG cells, pulse chase experiments were performed to estimate the half-life of the different forms of ectopically expressed BRK/Sik. Transfected NMuMG cells were metabolically labeled with [^{35}S] methionine for 1 hour, lysed at various chase times, and BRK/Sik was immunoprecipitated from total cell lysates. Newly synthesized kinase defective BRK/Sik K-M was more rapidly degraded than wild-type and activated BRK/Sik (Y-F), with the levels of labeled protein reaching equilibrium after a 6 h chase period (Figure 4). An increased rate of BRK/Sik K-M protein turnover suggests that

differences in protein expression levels between the different constructs may be attributable to decreased BRK/Sik K-M protein stability. These studies were performed in the nontransformed murine mammary gland cell line NMuMG. Experiments are ongoing to determine in BRK/Sik is also unstable in cancer cells.

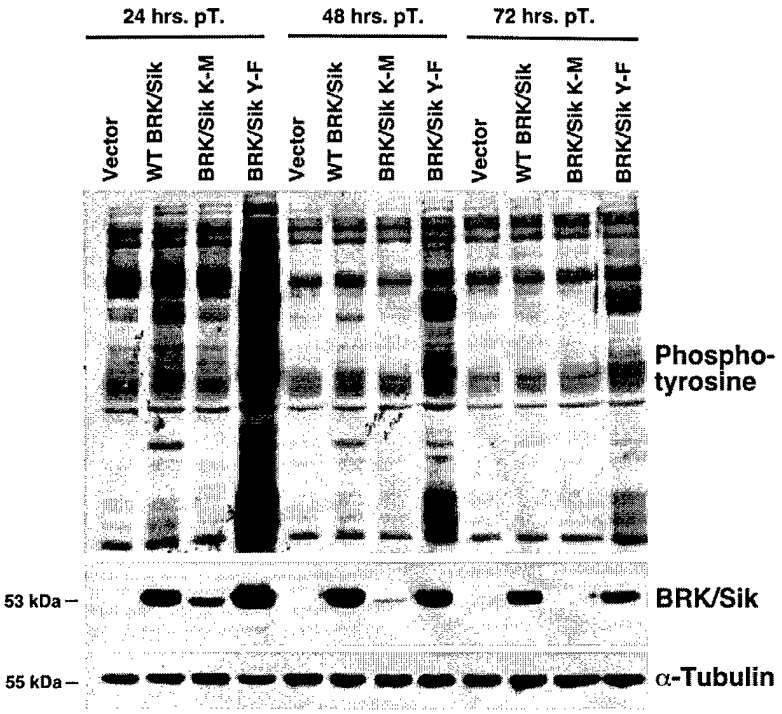

Figure 3. Ectopic expression of BRK/Sik is not maintained in normal murine mammary gland cells. NMuMG cells were transfected with equal amounts of the empty expression vector pcDNA3, wild-type BRK/Sik (wt Sik), kinase defective dominant negative BRK/Sik (Sik K-M) and constitutively active BRK/Sik (Sik Y-F). Total cell lysates were prepared and immunoblotting was performed with antibodies specific for phosphorylated tyrosine residues, BRK/Sik and α-tubulin as a standard. Cells containing the active form of BRK/Sik showed an increased level of phosphorylation compared to cells expressing WT BRK/Sik, with the phosphorylation as well as expression levels decreasing over time post transfection. Cells transfected with the kinase dead form of BRK/Sik showed a very weak expression level of this mutant suggesting, that BRK/Sik K-M is less stable than WT BRK/Sik or BRK/Sik Y-F.

Preliminary studies in the Rat1A fibroblast cell line indicate that active forms of BRK/Sik induce apoptosis (Figure 5). Following serum starvation, increased apoptosis was observed in cells transfected with wildtype and activated myristoylated and nonmyristoylated BRK/Sik. While this increase was not observed in cells transfected with the inactivated KM construct, expression studies revealed that KM was not maintained in selected cells. We have been able to isolate Rat1A and NMuMG stable cell lines expressing the different BRK/Sik isoforms, these cell lines lose BRK/Sik expression/activity even while under selection. We are currently developing

BRK/Sik inducible systems to be able to tightly modulate BRK/Sik induction and determine immediate consequences of BRK/Sik expression in normal murine mammary gland epithelial cells. Studies are currently underway to examine how breast cancer cells that express high levels of BRK/Sik avoid growth arrest or apoptosis. These studies may provide additional insight about significance of BRK/Sik functions in breast tumors.

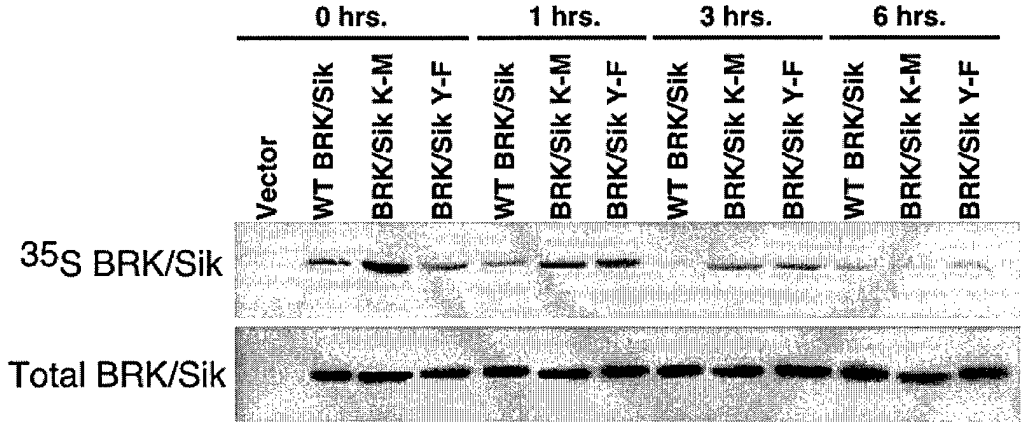

Figure 4. BRK/Sik protein turnover in NMuMG cells. NMuMG cells were transiently transfected with equal amounts of the expression vector pcDNA3, wild-type BRK/Sik (wt Sik), kinase defective dominant negative BRK/Sik(K-M) and constitutively active BRK/Sik(Y-F). Transfected cells were metabolically labeled with [^{35}S]methionine for 1 hr, rinsed, and transferred to medium containing excess of unlabeled methionine for various amounts of time (chase). BRK/Sik was then immunoprecipitated and analyzed by SDS-PAGE and radiography. The K-M construct has the shortest half-life (~ 90 minutes).

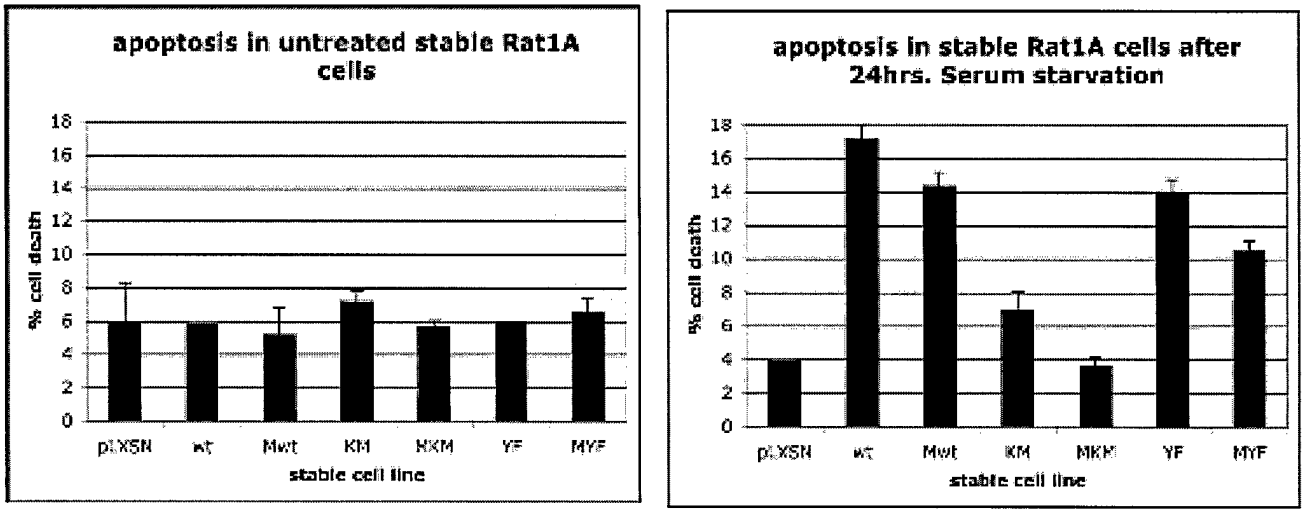

Figure 5. Active BRK/Sik induces apoptosis in Rat1A cells. Stable Rat1A polyclonal cell lines expressing the normal and myristoylated forms of wild-type BRK/Sik (wt, Mwt), kinase defective dominant negative BRK/Sik (KM, MKM) and constitutively active BRK/Sik (YF, MYF), as well as the control cell line expressing the vector pLXSN alone, were established using retrovirus infection. Selected populations were plated at equal density and apoptosis was induced by serum starvation in 0% FBS for 24 h. As a control cells were grown for the same time in 10% FBS. The percentage of cells with condensed chromatin was determined by DAPI staining. The data represents the average (± SE) of three independent

experiments. Stable cell lines expressing wildtype or the activated form of BRK/Sik (unmyristoylated as well as myristoylated) undergo more severe apoptosis than cells expressing vector alone or the kinase dead form of BRK/Sik.

KEY RESEARCH ACCOMPLISHMENTS

The major objectives of the funded proposal were to gain a better understanding of the roles of the BRK/Sik RNA-binding substrates in the etiology of breast cancer Our specific aims and the results/conclusions are summarized below:

Aim 1) To characterize the expression of STAR family RNA-binding proteins in normal breast tissue and breast cancer cells. We determined that SLM1 and SLM2 are novel STAR family substrates. Of the three STAR family substrates of BRK/Sik, we found that only Sam68 is expressed at significant levels in both normal mammary gland epithelial cells and breast cancer cells.

Aim 2) To test the hypothesis that phosphorylation of RNA-binding proteins by BRK/Sik correlates with the development of breast cancer. The STAR family substrates are involved in regulating RNA metabolism (splicing, transport, translation) [31]. We found that BRK/Sik phosphorylation of the three STAR protein substrates Sam68, SLM1, and SLM2 leads to the inhibition of the RNA-binding activities of the different STAR substrates in transfection studies. Because of its relatively high levels of expression in mammary gland cells, we focused on Sam68 in subsequent studies, and no notable difference in endogenous Sam68 tyrosine phosphorylation in BRK/Sik +/+ and -/- cells has been detected. In normal cells other kinases may also be able to phosphorylate Sam68 in the absence of BRK/Sik. However, strong induction of BRK/Sik in breast tumors could lead to alterations in posttranscriptional regulation of gene expression.

Aim 3) To test the hypothesis that BRK/Sik activity modulates gene expression in breast cancer cells by STAR protein-dependent and independent mechanisms. We have determined that oncogenic functions of BRK/Sik in breast cancer cells may not depend on phosphorylation of the STAR family substrates. Sam68 is expressed at high levels in normal cells and cancer cells. Induction of BRK/Sik in breast tumors may lead to enhanced phosphorylation of Sam68 and activation of growth promoting signaling pathways. Alternatively, BRK/Sik oncogenic

functions may depend on its association with inappropriate signaling proteins such as members of the ErbB (EGFR) receptor family.

SOW

Task 1: BRK/Sik is not expressed in the normal mammary gland at any stage of development, but it is induced in breast tumors. We determined that of STAR protein substrates, only Sam68, but not SLM1 or SLM2, is expressed during normal mammary epithelial cell development in the mouse using immunoblotting and immunohistochemistry.

Task 2: We found that BRK/Sik and Sam68 is expressed in breast tumor cell lines. However endogenous BRK/Sik and Sam68 do not show complete colocalization in tumor cells. We demonstrated that phosphorylation of nuclear STAR protein substrates is important for BRK/Sik nuclear localization.

Task 3: IN PROGRESS.

Task 4: IN PROGRESS.

Task 5: IN PROGRESS.

Task 6: IN PROGRESS.

Task 7: We determined that BRK/Sik phosphorylation of the STAR proteins Sam 68, SLM1, and SLM2 leads to inhibit of their RNA-binding activities in normal NMuMG cells.

Task 8: Lack of co-expression and colocalization suggest that BRK/Sik regulated modification of STAR proteins does not play a primary role in breast cancer cells.

In addition to the goals outlined in the SOW, we determined that BRK/Sik protein is not stable in normal murine mammary gland cells, and that overexpression of different BRK/Sik isoforms leads to an increase in apoptosis in nontransformed cells. In the future it will be important to determine how breast tumor cells maintain high levels of BRK/Sik expression and resist BRK/Sik induced apoptosis.

REPORTABLE OUTCOMES

1. **Manuscripts**

Haegebarth A., Heap, D., Bie W., Derry J.J., Richard S., and Angela L. Tyner. The Nuclear Tyrosine Kinase BRK/Sik Phosphorylates and Inhibits RNA-binding Activities of the Sam68-like mammalian proteins SLM1 and SLM2. In preparation.

Serfas M.S. and **A. L. Tyner**. 2003. Brk, Srm, Frk, and Src42A form a distinct family of intracellular Src-like tyrosine kinases. Oncology Research **13**, 409-419. [9]

2. **Presentations at National Meetings**

Haegebarth A and A. L. Tyner. Overexpression of the Intracellular Tyrosine Kinase Sik (Brk/Ptk6) Induces Apoptosis in Rat1A Cells. Accepted for presentation at the Cold Spring Harbor Conference, "Cancer Genetics & Tumor Suppressor Genes," August, 2004.

Haegebarth A., Heap D., Bie W., Richard S. and A. L. Tyner. Newly identified nuclear substrates of the epithelial specific tyrosine kinase Sik (BRK/PTK6). Presented at FASEB Summer Conference, "Growth Factor Receptor Tyrosine Kinases in Mitogenesis, Morphogenesis, and Tumorigenesis. August 2-7, 2003.

Heap D., Bie W., Kim E.J., Park J.H.Y., Richard S., and A. L. Tyner. 2003. The RNA-binding proteins SLM1 and SLM2 are substrates of the epithelial tyrosine kinase BRK/Sik. Gastroenterology 124 (4) 535, A66. Presented at the American Gastroenterological Association Digestive Disease Week, 2003.

Tyner A.L., Richard S., Derry J.J., Ye X., and W. Bie. The breast tumor kinase Brk is a nuclear tyrosine kinase that phosphorylates and regulates the RNA-binding protein Sam68 and the Sam68-like mammalian proteins SLM-1 and SLM-2. Presented at the 2002 ERA OF HOPE Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting in Orlando, Florida.

CONCLUSIONS

Our experiments led to the identification new specific substrates of the breast tumor tyrosine kinase BRK, and will lead to a better understanding of signal transduction pathways regulated by BRK in breast cancer. Thus far Sam68 is the only BRK/Sik substrate expressed at significant levels in the normal mammary gland and in breast cancer cells. Thus our data indicate that Sam68 should be the focus of future studies onthe roles of STAR proteins in breast cancer.

In transfection experiments we found that BRK/Sik efficiently enters the nucleus where it can phosphorylate and inhibit the RNA-binding activities of the three identified STAR protein substrates Sam68, SLM1, and SLM2. We have determined that mutation of the carboxy-

terminal tyrosine of BRK/Sik not only prevents negative regulation of the enzyme by tyrosine phosphorylation, but may also alter intracellular localization of a pool of the protein.

RNA metabolism plays an important role in growth control. Sam68 has been implicated in the regulation of RNA splicing, transport and translation and may be an important regulator of normal cell growth [31]. Aberrant expression and/or phosphorylation or lack of phosphorylation of Sam68 may contribute to the development of breast cancer. These proteins may provide new prognostic indicators for breast cancer and be potential targets for therapeutic intervention.

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APPENDIX 1

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OVEREXPRESSION OF THE INTRACELLULAR TYROSINE KINASE SIK (BRK/PTK6) INDUCES APOPTOSIS IN RAT1A CELLS

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Sik (BRK/PTK6) is an epithelial-specific non-receptor tyrosine kinase expressed in skin and throughout the gastrointestinal tract. While expression of Sik is restricted to differentiating cells of these epithelial linings, Sik expression is also upregulated in epithelial tumors. We previously demonstrated that Sik can enter the nucleus where it phosphorylates and inhibits the KH-domain containing RNA-binding protein Sam68, which plays important roles in RNA metabolism associated with growth. To identify mechanisms by which Sik may regulate both differentiation and oncogenesis, we have generated a series of Sik expression constructs that alter the activity of expressed protein in vitro and in vivo. These expression constructs include wildtype, constitutively activated (Y447-F) and kinase defective (K219-M) forms of Sik targeted to the nucleus or the membrane. Polyclonal Rat1A fibroblasts stably expressing these constructs were established and their growth characteristics were analyzed. We found that overexpression of active Sik (WT and Y-F) resulted in a reduced number of cells compared with cells transfected with vector or kinase dead Sik. A decrease in cell number could result from reduced proliferation or increased apoptosis. To explore the roles of the Sik kinase in apoptosis, we examined the response of our stable Rat1A cell lines to apoptosis induced by growth factor withdrawal and ultraviolet irradiation. Following serum deprivation and/or UV exposure, cells were fixed and DAPI staining was performed to evaluate the number of cells with condensed chromatin. Higher levels of apoptosis were detected in stable cell lines overexpressing an active form of Sik (Wt and Y-F) compared to cell lines expressing vector alone or kinase dead BRK/Sik. Furthermore, apoptosis also occurred earlier in these cell lines. These results suggest that Sik may regulate cell survival in vitro. Studies to determine in vivo functions of Sik in wildtype and knockout mice are underway.

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APPENDIX 2

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Brk, Srm, Frk, and Src42A Form a Distinct Family of Intracellular Src-Like Tyrosine Kinases

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The tyrosine kinases Brk/PTK6/Sik, Srm, Frk/Rak/Gtk/Iyk/Bsk, and Src42A/Dsrc41 have a low degree of sequence homology to other known kinases, including one another. We show here that the exon structure of these kinases, which we will call the Brk family, is highly conserved and distinct from each of the major families of intracellular kinases containing SH3, SH2, and tyrosine kinase domains, including c-Src and Fyn. Brk/Sik and Srm are 1.1 kb apart on human chromosome 20q13.3 and likely are the result of duplication in *cis*. Several Brk family kinases have an inhibitory effect on Ras pathway signaling from receptor tyrosine kinases. Members of this family can act either in the membrane or at the nucleus, and may change localization patterns depending on external stimuli. Brk has been shown to phosphorylate two proteins in vivo: Sam68, a substrate for Src in mitosis that can substitute for Rev in nuclear export of RNAs; and BKS, a novel adaptor molecule. Brk also functions as a rapid downstream signaling intermediate following calcium-induced differentiation in keratinocytes. It is possible that Brk family kinases may share common functions and interaction partners, which remain for the most part unexamined.

Keywords: BRK; Sik; Srm; Frk; Rak; Src42A

The pp60src transforming protein of the Rous sarcoma virus was the first protein found to have tyrosine kinase activity (1). Its endogenous homologue Src was soon found to be present in vertebrates (2), was sequenced (3), and was determined to lack transforming activity in a viral context (4). Association of pp60src with the membrane was shown to be the result of posttranslational lipid modification at the amino-terminus of the protein (5). Accordingly, Src has become the prototype of a large family of membrane-bound intracellular tyrosine kinases that share a myristoylated amino-terminal unique domain, SH3 and SH2 protein association domains, a single C-terminal tyrosine kinase domain, and a variable regulatory C-terminus, which is subject to a regulatory tyrosine phoshorylation pathway by the related tyrosine kinase Csk, which arose early in metazoan evolution (6).

Src42A (Dsrc41) was cloned as a putative *Drosophila* Src homologue (7) and as Su(Raf)1, a suppressor of partial loss of Raf function (8). By sequence homology and the presence of two conserved intron positions, it is more similar to vertebrate Src than any other *Drosophila* gene. Like vertebrate Src, it modulates signaling dependent on the epidermal growth factor receptor (EGFR[3]), Ksr, Raf, Ras1, and the rolled MAP kinase, but surprisingly, the role of Src42A is generally inhibitory in this context (9,10). It can also signal via JNK kinase activity (11). Src42A is weakly expressed in all cells at the cellular blastoderm stage and in a wide range of tissues during embryonic development. Its functional roles include modulation of dorsoventral polarity in the egg, dorsal closure of the embryo, and ommatidial organization of the third instar eye disc.

A fragment of the human tyrosine kinase BRK (breast tumor kinase) was initially cloned from cultured normal human melanocytes using reverse transcription PCR with degenerate primers corresponding to the conserved regions of tyrosine kinase catalytic domains and named PTK6 (protein tyrosine kinase 6) (12). A portion of the catalytic domain coding sequence was subsequently cloned using reverse transcription PCR and RNA isolated from involved axillary nodes from a patient with metastatic breast cancer (13). BRK cDNA clones were also isolated from a normal human small intestinal cDNA library (14). While BRK is most closely related to members of the Src family, it is highly diverged, with nearly equivalent homology to the proto-oncogenes c-Src, p60-YRK, p59-FYN, c-YES, and FRK/RAK with 44–45% protein identity. The BRK gene has only two intron boundaries conserved with the SRC family members (15,16), suggesting that BRK is part of a distinct family of nonreceptor tyrosine kinases.

BRK is expressed in normal human esophagus, stomach, duodenum, and colon epithelia (14). It is also expressed in breast and colon tumors and in breast, colon, and metastatic melanoma tumor cell lines (13,14,17,18). No expression of BRK has been detected in the normal mammary gland (14,17). Thus far, the sequence of BRK isolated from tumor cells and normal cells appears identical (15,16), suggesting that BRK, which is overexpressed in tumor cells, is the normal protein.

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[3]Abbreviations used: EGFR, epidermal growth factor receptor; BRK, breast tumor kinase.

The mouse orthologue of BRK is the Src-related intestinal kinase Sik, which was originally identified in a screen for tyrosine kinases in intestinal epithelial cells (19). Although the functional domains of BRK and Sik are conserved, Sik and BRK share only 80% amino acid identity, and 83% nucleotide identity. Southern blotting and gene mapping experiments confirmed that Sik and BRK are orthologues. The mouse Sik gene maps to the distal end of mouse chromosome 2 in a region that shares conservation of synteny with human chromosome 20q13, where the BRK gene has been mapped (14). Expression of Sik has been detected in skin and all linings of the gastrointestinal tract (20).

Srm was cloned by PCR amplification from neural precursor cells of 10-day mouse embryos, and is expressed in adult lung, testes, and liver at highest levels, in spleen, kidney, and ovary at moderate levels, and at low levels in intestine, muscle, thymus, heart, cerebellum, and cerebrum (21). Within the brain, Srm is expressed uniformly in cerebellum and cerebrum from embryonic day 15 to adulthood, but not in embryonic day 11 brain or in cultured neural precursor.

FRK/RAK was cloned from the human B-cell lymphoma cell line BL979 and shown to be present at high levels in the Hep3B liver cell line (22). Additionally, it was cloned from the BT-20 human breast cancer line, and found to be expressed at high levels in epithelial cell lines including BT-20 and the LS180 colon cancer human cell line (23). Expression of FRK mRNA was found in kidney, pancreas, liver, and skeletal muscle, and was absent from lung. Protein was detectable at high levels in the liver, and to a lesser extent in the kidney, but not in lung, muscle, brain, or heart. By autophosphorylation assay and antiphosphotyrosine immunoprecipitation it was shown to possess intrinsic tyrosine kinase activity. Quantitatively, in an amplification from degenerate primers FRK represented 15.8% of 2000 clones from the renal carcinoma line RCC23, but only 0.7% from the esophageal cancer line TE1 (24). Overall, FRK transcripts were detected in five of five renal cancer lines, one of three gastric cancer lines, but not in esophageal, sarcoma, or lung cancer lines.

The murine homologue of FRK (Iyk/Bsk) has been cloned from pancreatic islet cells (25). By Northern analysis, it is expressed in kidney, liver, lung, and skeletal muscle, but is absent from brain, spleen, testis, and heart. By RT-PCR, expression in islet cells from adult mouse, fetal rat, or the RINm5F cell line greatly exceeded that in kidney. Mouse FRK was also cloned from mature resting mammary glands (26) and shown to be present at highest levels in intestine and to a lesser extent in ovary, placenta, kidney, and day 13 embryo, with comparatively undetectable levels in liver, lung, skeletal muscle, brain, spleen, testis, heart, skin, and thymus. Within the intestinal tract, expression was highest in the ileum and jejunum, with low levels in the cecum and comparatively undetectable levels in colon, salivary gland, pancreas, and stomach.

The rat homologue of FRK (Gtk) has been cloned from small intestine, and transcripts are highly expressed in intestine with six- and eightfold lower levels in kidney and liver, respectively (27). By ribonuclease protection analysis Gtk is expressed most highly in rat intestine, followed by pancreas, kidney, liver, and lung, and not detectable in leg muscle, brain, spleen, or heart. Within the rat intestinal tract, Gtk was detected at highest levels in ileum, moderate levels in stomach, duodenum, and jejunum, and low levels in colon. To facilitate comparisons between species, the names Brk, Frk, and Srm will be used to describe mouse, rat, and human orthologues of each gene throughout the remainder of the text.

RESULTS AND DISCUSSION

Evolution of Brk Family Kinases

Sequence homology is not in itself sufficient to associate any of the proteins Brk, Srm, Frk, or Src42 with one another. Typically, these kinases have been classed separately from the known intracellular tyrosine kinase families (28,29). In Figure 1, these kinases and others representing the established families of kinases containing an SH3–SH2–tyrosine kinase domain structure are aligned pairwise. Clearly, such kinases as Abl, Csk, Srm, and Brk form comparatively poor alignments with all the others selected. Homology searches and trees assuming a constant clock rate of divergence may be deceiving, because these kinases are either diverging more rapidly than the others or have fewer constraints on where or how far they are able to diverge from the others. Even where homology can be inferred from the data, such as with Brk and Srm, the margin by which this alignment surpasses the others is only a few percent.

However, genomic sequence has become available (AL121907.20, AL121829.29) that links human Brk within four kilobases of a diverged Src-like kinase with similar structure, Srm at chromosome 20q13.3 (Fig. 2). Close linkage of the genes is confirmed by mapping of the murine orthologues of each to distal chromosome 2 (14,21). The human Srm has not been cloned as a cDNA, but if nonhomologous EST transcripts appropriately oriented and polyadenylated extending to 2329 bp following the conserved stop codon are assumed to represent Srm transcripts, the distance between the presumed polyadenylation site of Srm (AA534748.1, AI762098.1) and the initiation site of Brk (20) is only 1112 bp. The genomic sequence also establishes that Srm and Brk share a precisely conserved exon structure, with seven introns at identical positions in each transcript. Final genomic sequence of Frk has been made available (AL121963.10) and shares identical intron placement with Brk and Srm. The arrangement of intron positions relative to a consensus sequence for the intracellular tyrosine kinases is shown in Figure 3.

The relationship between the exon structure of Brk and Src42A has been established, with at least six of seven Brk splice junctions well conserved (15,16). Two additional introns exist in Src42A, and a seventh intron position varies by only three amino acids, which surround a central intron position found in BTK29A. For Figure 3, intron positions within two amino acids of one another were grouped, and thus all seven intron posi-

	SRC64B	ABL	TXK	BTK29A	SRC	CSK	F49B2.5	SRM	FRK	BRK	SRC42A	FYN	STK	SRK1/4
SRC64B		41	36	39	53*	33	45	40	47	43	50*	48	51*	48
ABL	41		37	39	42	38	40	38	43	36	43	39	45	44
TXK	36	37		50*	38	35	33	34	35	32	39	36	37	37
BTK29A	39	39	50*		40	36	38	35	38	34	40	39	41	39
SRC	53*	42	38	40		39	55*	42	50*	44	59*	69*	66*	56*
CSK	33	38	35	36	39		37	34	36	36	38	35	35	37
F49B2.5	45	40	33	38	55*	37		42	53*	39	65*	51*	55*	56*
SRM	40	38	34	35	42	34	42		42	46	45	41	41	43
FRK	47	43	35	38	50*	36	53*	42		43	61*	48	53*	56*
BRK	43	36	32	34	44	36	39	46	43		45	40	42	43
SRC42A	50*	43	39	40	59*	38	65*	45	61*	45		54*	61*	61*
FYN	48	39	36	39	69*	35	51*	41	48	40	54*		58*	49
STK	51*	45	37	41	66*	35	55*	41	53*	42	61*	58*		59*
SRK1/4	48	44	37	39	56*	37	56*	43	56*	43	61*	49	59*	

Figure 1. Homology of widely diverged kinases containing the SH3–SH2–YK structure. Kinase domains from mammalian representatives of the Abl, Csk, Src, Tec families, three diverged Src-like kinases from *Drosophila* (BTK29A, Src64B, Src42A), and Src-like kinases from nematode (F49B2.5), *Hydra attenuata* (STK), and sponge (SRK1/4) were chosen for alignment. To minimize the effects of divergent N-terminal regions, homology was calculated from the first SH3 motif to the C-terminal tail, or in the case of Abl the 3′ exon–exon boundary of the kinase domain. The percent amino acid identity from paired gapped alignments is shown, and homologies above 50% are marked by asterisks. A bracket indicates the sequences that are proposed to represent the Brk family.

tions of Brk were considered equivalent to Src42A in this alignment.

Representatives of each of the recognized intracellular tyrosine kinase families (Src, Tec, Abl, Csk) (28) containing SH3, SH2, and tyrosine kinase domains, for which genomic sequence had been made available, were chosen for alignment. Within the Src family, in addition to Src, Fyn was chosen for alignment as it possesses greater protein homology (49%) with Frk (22) than exists between Frk and Brk (44%). The intron positions of Src and Fyn vary substantially and are separated by highly conserved amino acid sequence, but each is the prototype for other Src family kinases: Hck, Lck, and Fgr with Src (30), and Yes with Fyn (31). Additionally, the other *Drosophila* Src homologues at 29A and 64B and kinases homologous to the Brk family from *Hydra* and *Spongilla* were considered. Of these, the best match was with STK and Txk with only two of seven introns conserved. Consideration of very limited information regarding Srk1 indicates that it is, at least, not a perfect match.

Consideration of the intron positions seems to defy

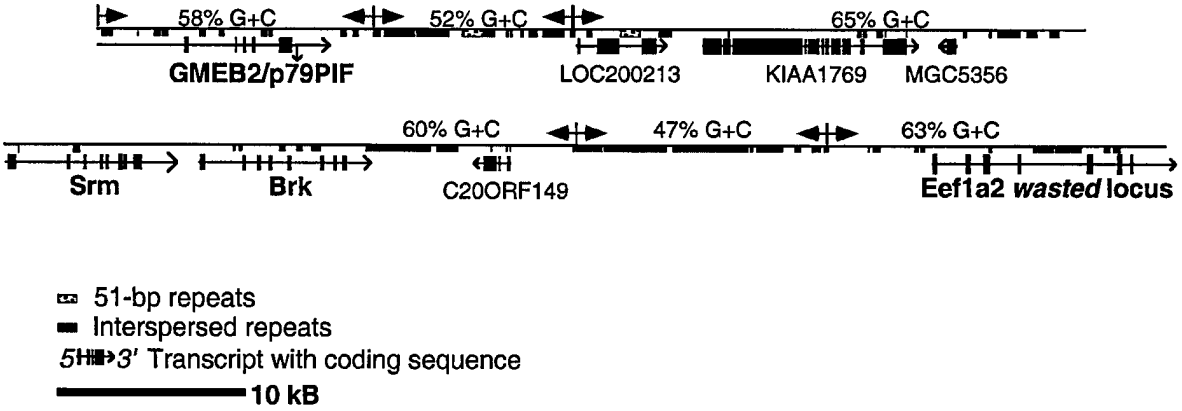

Figure 2. Tight linkage of Brk and Srm on chromosome 20. Known and predicted protein coding transcripts from human chromosome 20q13.3 are diagrammed. The positions of repeat elements predicted by RepeatMasker (Smit, AFA & Green, P. RepeatMasker at http://ftp.genome.washington.edu/RM/RepeatMasker.html) are marked above. Two large arrays of near-perfect 51-bp tandem repeats (stippled) are not homologous to one another. The region is arbitrarily divided into segments of varying G + C content as marked at top, which reflect a large difference between repetitive and gene-rich regions. Unrelated linked genes include those encoding the eukaryotic translation elongation factor 1a2 (Eef1a2, mouse wasted locus) (58) and the glucocorticoid modulatory element binding protein 2 (GMEB2) (59).

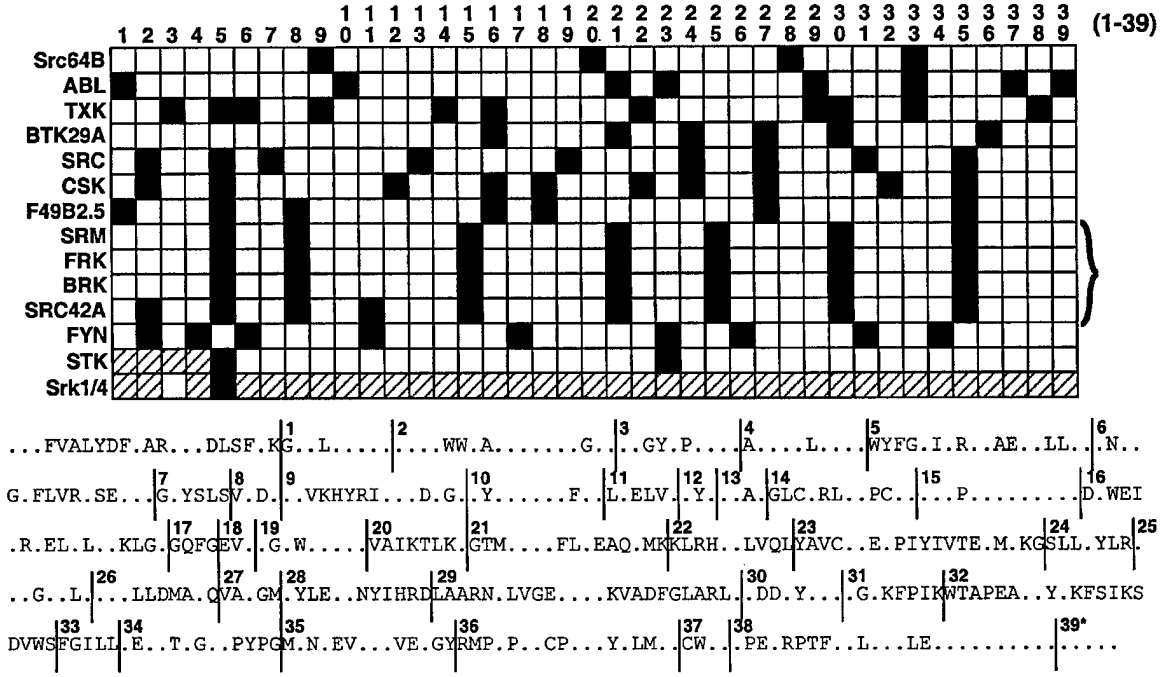

Figure 3. Intron positions of diverged intracellular kinases. An alignment of the conserved regions from Figure 1 was used to generate a consensus sequence (bottom), and intron positions relative to the predicted protein sequence are indicated by black squares at the corresponding numbered positions. A bracket marks the sequences that are proposed to represent the Brk family.

attempts to draw a simple phylogenetic tree, either of intron insertion or loss. One example is provided by the first intron of Src42A, which is absent from Brk, Srm, or Frk, but closely conserved with Src, Fyn, and Csk. Comparison of sequences immediately surrounding this intron (KGEHLEILNDTQ*GDWWLAR) suggests a greater kinship with Src (KGERLQIVNNT*EGDWWLAH) than with the Brk (RAGDVFHVARKEEQWWWATL) family kinases, suggesting that either Src42A or the common ancestor of the vertebrate Brk family was subjected to an illegitimate recombination or gene conversion event that added or removed an intron while altering the surrounding coding sequence. More generally, expansion of the tyrosine kinase family predates the origin of multicellular animals (32). If the unicellular organisms in which the initial duplications of tyrosine kinases occurred possessed efficient homologous recombination, it is possible that conserved sequences and included introns could have been interchanged between the diverging genes.

Comparison of the structures of the Brk family kinases (Fig. 4) indicates that there has been some conservation of the intron structure. For instance, the sets of exons 2, 3, 4 and 6, 7, 8 remain clustered in each primary transcript. Primate-specific Alu elements account for most of the intron expansion of the human genes, while insertion of a region including the hypothetical *Drosophila* protein CG14471 may account for most of the expansion of Src42A.

It is interesting that Brk and Srm have lower overall homology to non-Brk family kinases (Fig. 2). This could be a consequence of their recent duplication, or may be related to their position in the genome. These genes have been mapped close to the telomere at 20q13.3, and are in a large region of high G + C content. Given that Brk is in a gene-rich high-melting region near the telomere of chromosome 20, it is likely to be localized to the T (temperature) band in this position. T bands may result from a bias in direction of gene conversion events between alleles, reflecting local recombination hotspots, and the processes by which they arose may have subjected Brk and Srm to a higher rate of mutation. Subsequently, G + C content is thought to be lost via methylation of CpG dinucleotides. Brk and surrounding genes have lost CpG and G + C content at different rates in humans and mice, so that the mouse orthologues have a G + C content in the coding region 9% lower than in humans, and have lost most of the CpG dinucleotides (14).

Another peculiarity of the G + C content in and surrounding Srm is the occurrence of interchanges of G- and C-rich sequence on one strand with a 50–60 base-pair periodicity (Fig. 5). Possibly as a result of these periodic variations in nucleotide content, plots of self-homology diagonals in Srm suggest many possible alignments extending over 200–400 bp regions, varying in alignment position from one another in increments of 50–60 bp. This is seen in the promoter region (Fig. 5) as well as in the first and second introns (not shown), but not in Brk promoter or intron sequences. While most such alignments are very poor, not exceeding 50% nu-

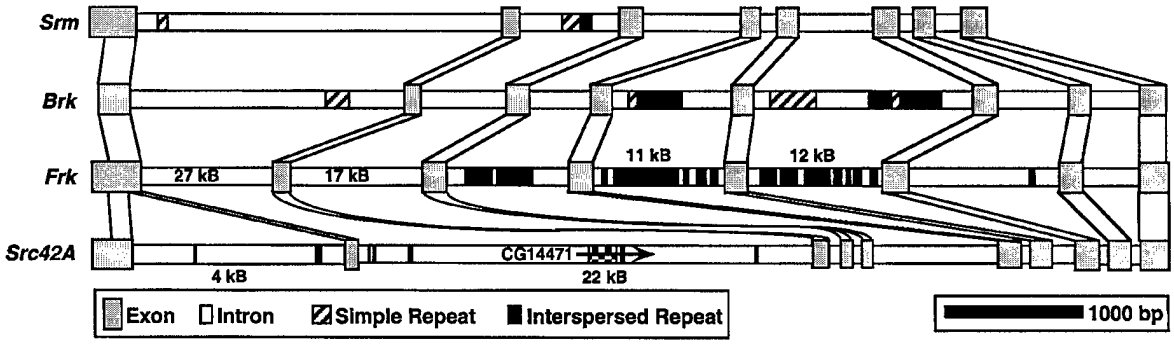

Figure 4. Comparison of the exon structures of Brk family members. Homologous exons and introns are drawn to scale except as otherwise marked. Simple repeats (excluding low complexity sequence) and interspersed repeats predicted by RepeatMasker are shown (Smit, AFA & Green, P. RepeatMasker at http://ftp.genome.washington.edu/RM/ RepeatMasker.html). Contiguous sequence for the first two introns of Frk was not available, so these repeats are not marked. The hypothetical protein CG14471 is located in the long second intron of Src42A, perhaps reflecting the reverse transcription and insertion of a gene or pseudogene. Much of the remaining intron length variation can be accounted for by recent insertions of primate-specific interspersed repeats, which may have inserted more frequently into Frk because of its lower G + C content.

cleotide identity, there are much better conserved tandem repeats 40–60 bp in length found at various positions up to 30 kb upstream of Srm. However, each such repeat region has a unique sequence without detectable homology with the others. One hypothesis to explain these features is that systematic trends of mutation may have been imposed in the Srm region based on features of chromatin structure that created the alternating nucleotide content, eventually creating a few regions 50–60 bp apart with sufficient homology that illegitimate recombination events could expand random repeats with this periodicity.

Targeted Disruptions of the Brk Family

Homozygous deletions of Srm in mice have been produced, with no detectable phenotypic effect (21). While consequences of disruption of the Brk gene in the mouse are under investigation, Brk-deficient animals are viable, fertile, and lack obvious physical deformity (Tyner and colleagues, unpublished data). Mice deficient for Frk also exhibit only a mild phenotype, and are viable and not prone to developing spontaneous tumors (33). However, loss of function alleles of Src42A causes homozygous lethality in *Drosophila* (34) and can be complemented by ectopic expression constructs of Src42A, but not c-Src. Double mutants showed defects in head involution and tail morphology, and died from progressive tracheal necrosis as indicated by a brownish coloration, either before hatching or during the first instar larval stage. The lack of comparable effects for the individual mammalian Brk family members suggests that they may share redundant functions.

CSK Regulation

In the Src family, CSK phosphorylation inhibits kinase activity by promoting an intramolecular interaction between the C-terminus and the SH2 and SH3 domains (35). BRK and Sik have conserved tyrosines at position 447 that correspond to tyrosine 527 of Src. Mutation of tyrosine 447 in Sik to phenylalanine resulted in increased Sik kinase activity and phosphorylation of its substrate Sam68 (36). This indicates that Sik is negatively regulated by phosphorylation of the carboxy-terminal tyrosine at position 447 and that substitution of this tyrosine produces a more active kinase. Interestingly, overexpression of BRK with mutation of its tyrosine at position 447 resulted in reduced anchorage-independent growth of mammary epithelial cell lines and NIH 3T3 fibroblasts (37).

Frk has been shown to be subject to phosphorylation by CSK, which is specifically blocked by mutation of the most C-terminal tyrosine 497 (23). However, this tyrosine occurs in a very different sequence context from the regulatory tyrosine of Src, and the effect of this modification on Frk function has not yet been established. The *Hydra attenuata* gene STK has also been shown to be a substrate for a strong Csk homologue from *Hydra vulgaris* (6). STK and this homologue are closely coexpressed in *Hydra* epithelia, and in yeast coexpression experiments STK activity was blocked by CSK in a manner dependent on its C-terminal tyrosine.

Myristoylation

Members of the Src family are typically myristoylated at the amino-terminus, a lipid modification often associated with membrane localization (38). A glycine at the second position of the amino acid sequence is considered to be essential for myristate conjugation. Src42A contains a glycine in this position, and an activated Src42A-LacZ fusion protein was found to colocalize with *D*E-cadherin at adherens junctions in the *Drosophila* eye disc. By contrast, the mouse and human forms of Brk and Srm lack this glycine. Immunohistochemistry of mouse and human Brk sometimes shows a small component with membrane or tight junction localization, but has also demonstrated tissue- or cell type-specific localization to the cytoplasm, cytoplasmic granules, perinuclear cytoplasm, nucleus, or nuclear granules (36). In mouse and human prostate tissue, Brk is localized

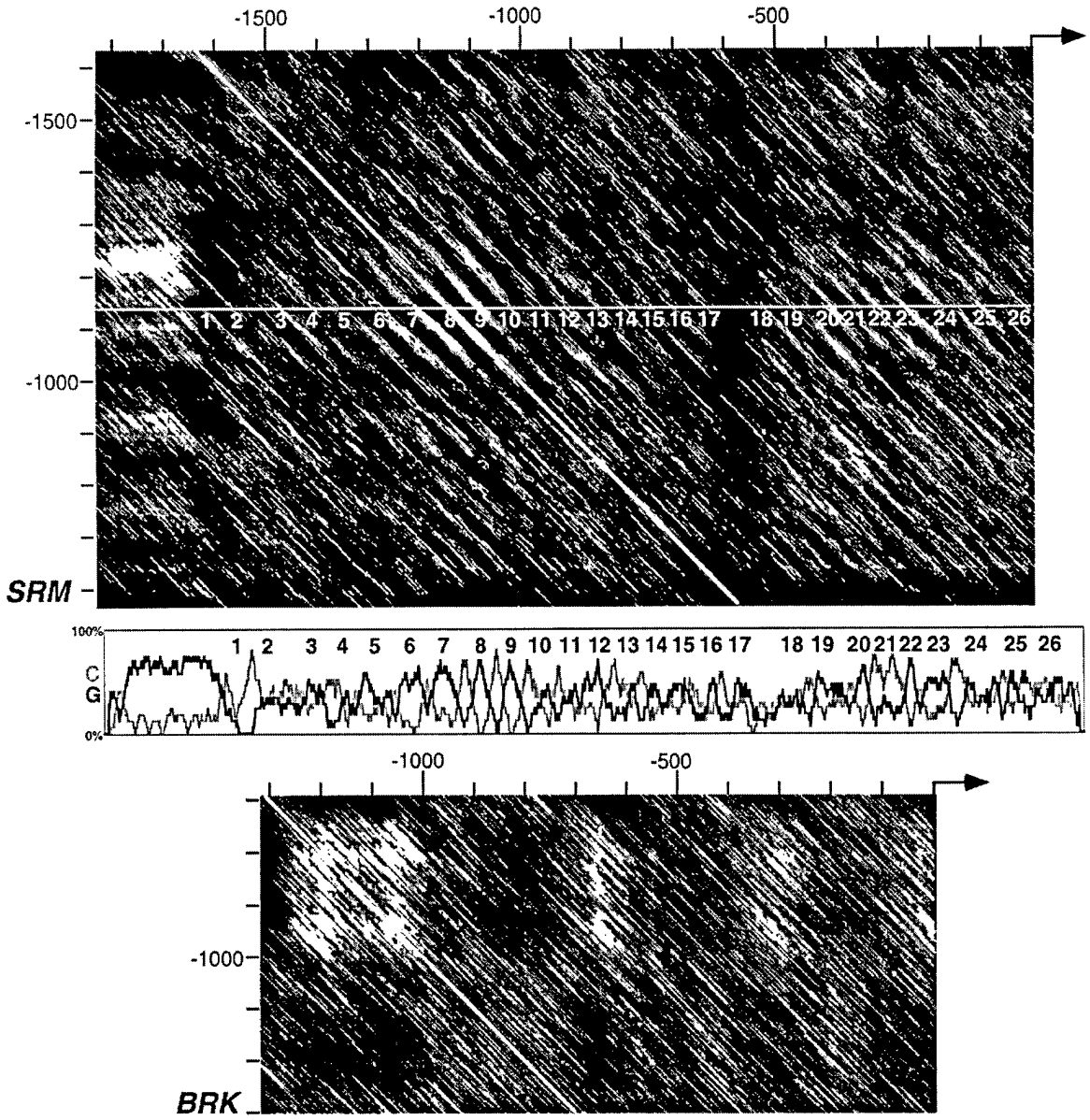

Figure 5. Regular patterning of the proximal Srm promoter. The GeneWorks program was used to plot self-homologous diagonals of greater than 35% homology in a 40-bp sequence. Output for top and bottom panels was converted to grayscale using Photoshop (Adobe), such that diagonals with 40% or better homology appear a brighter white. Interestingly, diagonals occur with regular spacing throughout the 2 kb of promoter and upstream sequence immediately 5′ of the Srm transcriptional start site (top). The horizontal line represents the alignment of one segment of the promoter with 26 regularly phased regions, generally 50–60 bp apart, in which diagonals are prominent. Although the G + C content of the region is relatively constant, when contents of G and C are analyzed separately as percentages of nucleotides within a 40-bp window, strong alternate peaks of G and C content (middle) correspond to the positions of these 26 alignments. The continuity of many diagonals over 400 bp or more (top) demonstrates that this periodic homology is present equally in G-rich, C-rich, and transitional regions, but the strength of this homology nonetheless correlates with positions where very high (>85%) content of either G or C is present. No periodic self-homology occurs in the sequence between the polyadenylation site of Srm and the transcriptional start of Brk (bottom).

to nuclei of epithelial cells (Derry and Tyner, unpublished data).

The human Frk protein also lacks glycine 2, and does not evidence membrane localization by immunohistochemistry (39) or subcellular fractionation (23). However, the mouse and rat Frk homologues contain a glycine-2 and one of two conserved serines of the consensus myristoylation (SH4) motif (26), though they lack three conserved lysine residues (25) and the serine 6 crucial for lck myristoylation (40). Despite these deficiencies, the rat myristoylation signal is clearly active, causing rat Frk to be labeled when transfected into Cos cells in the presence of [^{3}H]myristate and resulting in membrane localization confirmed by immunohistochemistry and subcellular fractionation (41). The localization of mouse Frk has not yet been examined, but the N-terminal sequence varies by only one amino acid from the rat protein. While the highly divergent intracellular localizations of human and mouse Frk invite speculation that additional family members could exist, they possess 88% protein homology (41) and 90% cDNA homology, and no candidate paralogues have emerged from genomic sequencing. While 5 of the first 10 amino acids differ between human and mouse Frk, the crucial Gly and Ser divergence requires but a single nucleotide transition (GGC vs. AGC).

Considering the lack of similarity between the myristoylation motif of mouse Frk and the Src family, and the ease with which the crucial glycine at position 2 may be lost or acquired, we should not expect myristoylation to remain a defining characteristic of a family of tyrosine kinases unless conserved protein associations require it. Indeed, even within the Src family, a form of Fgr translated from an internal AUG is found in the nucleus and cytoplasm, and Src itself can be found in the nucleus in keratinocytes undergoing calcium-induced in vitro differentiation (42). Because mouse and rat Frk are homologous with human Frk in the nuclear localization signal, it appears that they have either acquired myristoylation very recently, or that an as-yet unidentified nonmyristoylated splice form exists in nonintestinal tissues. It is also important to recall that myristoylation in itself is neither necessary nor sufficient for tyrosine kinases to associate with the membrane. For instance, the myristoylated splice form of Abl is localized primarily to the nucleus, although transforming mutants in which the SH3 domain has been deleted become activated with membrane localization (43).

Nuclear Localization and Protein Associations

Although nuclear localization has not been reported for Src42A, it is a common feature of the mammalian Brk family members. Nuclear localization of Brk has been detected in human breast and colon lines, mouse mammary and keratinocyte cell lines, primary cultured keratinocytes, and in prostate tissue. Mouse and human Brk associate with the RNA binding protein Sam68 in an interaction requiring Brk SH3 and SH2 domains and the proline-rich P3 region of Sam68 (36). In the transformed HT29 and MCF-7 adenocarcinoma cell lines, endogenous BRK and Sam68 colocalize in Sam68-SLM nuclear bodies (SNBs), while transfected Sik and Sam68 are localized diffusely in the nucleoplasm of nontransformed NMuMG mammary gland epithelial cells. Transfected Sik phosphorylates Sam68 in SNBs in HT29 cells and in the nucleoplasm of NMuMG cells. In functional studies, expression of Sik abolished the ability of Sam68 to bind RNA and act as a cellular Rev homologue (36).

Human, mouse, and rat Frk SH2 domains contain a putative bipartite nuclear localization signal (KRxxxxx FFxxRRR) from amino acids 168 to 181, in a position expected by analogy to the Src SH2 crystal structure to be accessible to other proteins (23). Accordingly, nuclear localization was investigated in COS7 monkey kidney cells. The majority of these cells restrict Frk to the nucleus as assayed by immunocytochemistry or subcellular fractionation. In the BT-20 human breast cancer line, substantial nuclear localization also occurs, but with a larger cytoplasmic component.

The nuclear localization of Frk in human breast epithelium has been shown to vary in accordance with the proliferation state of the tissue (39). In postmenopausal women, more than 50% of epithelial cells show strong nuclear staining in addition to cytoplasmic Frk signal. This proportion is decreased in tissue from the luteal phase of the menstrual cycle, and decreased further to less than 10% in the proliferating follicular phase. In a lactating adenoma, no nuclear signal was observed. In 21 of 21 invasive carcinomas, there was little or no nuclear signal, and reduced or absent cytoplasmic staining. These changes did not appear to occur as a direct result of systemic hormonal regulation, because tumor samples from postmenopausal women contrasted with immediately adjacent normal tissue with over 50% nuclear staining.

Based on its nuclear activity, human Frk was tested for association with pRb protein at the C-terminal "C pocket" previously shown to bind the Abl kinase domain (44). Frk also contains a sequence (EPYLPCLSTE) similar to the LxCxE motif of cyclin D1 (EHQLLCCEVE), although this sequence is not present in amino-terminal domains of the other Brk family members and is imperfectly conserved in mouse Frk. GST-Frk was shown to immunoprecipitate pRb from cell lysates of the SK-BR-3 breast tumor line, but not other SH3 binding proteins such as RasGAP, GAP-associated p62, and Shc. This interaction was confirmed to occur with endogenous Frk and pRb. Active Frk is bound to pRb, but pRb does not appear to be a substrate. Analysis of truncated fusion proteins indicated that both an Abl-like mechanism of binding between the kinase domain and the pRb C pocket, and an LxCxE-independent binding of the SH3 domain or near amino-terminal region to the A/B pocket are most important for full binding affinity. When the cells were arrested in G_1, S, or M, association was found equally in G_1 and S, indicating that Frk binds to pRb in a phosphorylation-independent manner.

Altered Expression in Proliferation and Tumor Progression

Increased Brk expression has been observed in a variety of tumors. Although it was cloned from normal melanocytes, Brk mRNA expression could not be detected

in these cells (12). In other studies Brk expression was not detected in two normal strains of melanocytes or in seven primary melanoma cell lines. In addition, Brk was not detected in biopsies from 10 metastatic melanomas (18). However, Brk mRNA and protein were detected in 2 of 22 metastatic melanoma cell lines (18).

The Brk cDNA was also cloned from a metastatic breast tumor and, by RNase protection, Brk transcripts were detected in one out of five grade III breast carcinoma RNAs, but not in two normal breast samples (13). By RT-PCR, Brk could be detected in three out of seven tumors but not in four normal breast RNA samples (17). Brk was surveyed in breast tumor cell lines by Western blotting and found at high levels in six, moderate levels in three, and low or zero levels in four. Of 41 primary breast tumor samples quantified by Western blotting relative to cytokeratin 18, Brk was overexpressed by fivefold or more in 27%, and overexpressed twofold or more in 61%, relative to normal breast tissue. One line expressed 43-fold higher levels of Brk protein.

While Brk is expressed in normal differentiating colonic epithelial cells, levels of Brk mRNA are higher in colon tumor samples when compared with adjacent normal tissue (14). Brk is expressed at high levels in the colon adenocarcinoma cell lines SW480 and HT-29 and to a lesser but still significant extent in T84 and Caco-2 cells. Levels of Brk expression in SW480 and HT-29 cells are equivalent to the levels expressed in the breast tumor cell lines MCF-7 and T-47D. These data suggest that Brk may play a role in signal transduction associated with the development of both tumor types. Brk mRNA levels have also been found at high levels in a small fraction of melanoma cell lines (18). In human tumors Brk is often relocalized from the nucleus to the cytoplasm (Derry and Tyner, unpublished).

Although an overall decrease in Frk expression was observed in breast carcinomas by immunohistochemical comparisons with normal tissue (39), increase in its expression has been observed by Western analyses (22) in which two of five colon cancers and two of six breast cancers overexpressed Frk. Interestingly, in one patient Frk overexpression was observed in a liver metastasis but not in the corresponding primary colon carcinoma or normal tissue. A more recent study, examining the tyrosine kinase expression profile in immortalized human breast epithelia cells and a number of breast cancer cell lines, identified both Frk (Rak) and Brk expression (45).

Modulation of Receptor Tyrosine Kinase Signaling and Other Growth and Differentiation Pathways

A Src42A loss-of-function mutant has been cloned as a suppressor of a partial loss-of-function mutant of Raf (34). In the *Drosophila* egg, Src42A mutants restored the production of dorsal appendages lost due to decreased Ras activity, and 10% of eggs laid by homozygous females of one strain were completely dorsalized. Because egg dorsalization is mediated by EGFR signaling, Src42A mutants were confirmed to suppress Sos loss of function mutation induced loss of wing veins, another EGFR-mediated effect. In the eye disc, hemizygosity for Src42A increases the rough phenotype caused by expression of constitutively active Ras1 or Raf. However, mitotic clones with homozygous loss-of-function mutation of Src42A do not result in a rough phenotype or extra R7 cells as are caused by activated Ras mutants. Additionally, in embryos lacking maternal Ras1 transcripts, Tor signaling for production of tll is decreased, but still present due to residual Ras1-independent activation of Raf. Deficiency of Src42A can partially compensate to increase tll and increase the rate with which certain embryonic structures are produced. Thus, wild-type Src42A appears to negatively regulate signaling from EGFR, Tor, and Sevenless receptors via Ras1/Raf and Sos signaling pathways.

Conversely, screening has been performed for mutations that suppress Src42A suppression of Raf null mutants (restoring Raf mutant-associated lethality) (10). A wide variety of known mutants were shown to have such activity, particularly intermediates in EGFR-mediated signaling. Screening for new EMS-induced mutants turned up primarily mutants in EGFR, rolled, and semang, a novel gene functioning in the EGFR pathway in eye and wing.

Src42A has also been shown to act on the hemipterous/basket pathway of dorsal closure, in which epidermal layers are signaled to elongate dorsally and fuse at the midline of the embryo (11). A Src42A mutant mimics hemipterous and basket mutants in inhibiting proper closure, which is a result of inhibition of a change in epithelial cell shape. Similarly, Src42A mutants decrease the downstream target puc in the wing disc, and, conversely, constitutively active Src42A causes ectopic puc expression. Hemipterous is a homologue of JNKK, and basket of JNK, and this pathway mediates signals from small GTPases such as Drac1, Dcdc42, and DrhoA to Djun and other phosphorylated transcription factors (46).

Stable expression of Brk in the Hb4a, a human mammary epithelial line, increases the sensitivity of the cell line to the mitogenic effects of EGF by a factor of two- to threefold (37). Binding of labeled EGF indicated that there was no increase in the number of EGF receptors present. Brk binding to EGFR was indicated by coimmunoprecipitation from MCF10-A cell lysates. While the morphology of transfected cells did not change, and no escape from contact inhibition was found, the number of colonies in soft agar was greatly increased. While a kinase motif mutant abolished this activity, interestingly the effect of mutation of the putative C-terminal CSK regulatory tyrosine was to substantially decrease colony formation. Brk expression in mammary epithelial cells has been shown to increase phosphorylation of ErbB3, a paralogue of EGFR, and subsequent recruitment of phosphoinositide 3-kinase, leading to greater PKB/Akt activation (47).

Brk has been shown to interact in vitro and in vivo with Sam68 (Src associated in mitosis, 68 kDa), a nuclear RNA binding protein (36). Sam68 was first identified as a substrate of Src during mitosis (48,49). Sam68 has been shown to bind RNA with UAAA motifs (50), and is localized in novel nuclear bodies called Sam68/SLM nuclear bodies (SNBs) in cancer cell lines (51).

Although the function of Sam68 is not well understood, Sam68 has been shown to be required for cell cycle progression (52), and can function as a cellular homologue of Rev by transporting unspliced HIV mRNA into the cytoplasm (53). Phosphorylation of Sam68 by Sik negatively regulated its RNA binding ability and its ability to function as a Rev cellular homologue (36). Phosphorylation of Sam68 by Brk within the nucleus could contribute to posttranscriptional control of gene expression during differentiation of epithelial linings or be necessary for proper growth control.

In primary mouse keratinocytes, exposure to high calcium concentrations results in an in vitro differentiation process (54). The kinase activity of mouse Brk is doubled within 2 min of this stimulus, and increases five- to sevenfold within 24 h. Activation of Brk is associated with binding of its SH2 domain to a tyrosine-phosphorylated residue on the GAP-associated p65 protein (which is distinct from Sam68) rapidly phosphorylated on tyrosine following calcium addition, but overexpression of wild-type or kinase-defective Brk does not affect the level of p65 phosphorylation. Overexpression of wild-type, but not of kinase-defective Brk, increases production of filaggrin, a cornification-associated protein representing the differentiation stage where Brk is usually expressed.

Recently, a substrate for Brk has been isolated by yeast two-hybrid screen, and found to be a novel protein, BKS, containing pleckstrin homology and SH2 domains, and candidate SH3 binding motifs (55). Unlike Brk, this protein is widely expressed in most tissues examined, suggesting similar interactions with other kinases. An intact SH2 domain of BKS is required for it to be a substrate for phosphorylation, but SH2 and SH3 domains of Brk have only minor influence on the interaction.

The interactions of Srm have not been well studied, as no published or commercial antibody against Srm of any species is reportedly available. A two-hybrid screen to identify SH2 domain fragments binding the IGF-1 receptor cytoplasmic domain yielded clones for Grb10, three isoforms of PI 3-kinase, Csk, Fyn, Src, and Srm (56). These clones were screened by verification that yeast cured of the LexA-IGF-I reporter plasmid lost expression, and that expression was regained following mating with a different strain carrying the reporter, a process that excluded half of the initial positive clones.

Rat Frk has also been shown to coimmunoprecipitate with the Met tyrosine kinase receptor, which transduces signals from hepatocyte growth factor (HGF). Met and Frk colocalize by immunoelectron microscopy, and through the use of DTBP for reversible cross-linking prior to immunoprecipitation their interaction was confirmed (57). HGF increases autophosphorylation of both Met and Frk within 10 min, and serum starvation of NIH 3T3 cells reduces Frk kinase activity to undetectable levels over 72 h. Hepatocytes isolated by collagenase perfusion exhibit a threefold increase in Frk activity 10 min after HGF stimulation.

As described above, human Frk has been shown to interact with pRb in the nucleus. Overexpression of a vector containing Frk in NIH 3T3 cells results in 60% decrease in the number of viable colonies, and surviving colonies had diminished Frk expression (44). Frk is expressed at higher levels in cells arrested in G_1 or S phases by serum starvation or hydroxyurea, suggesting that it may have a physiological role in mediating these processes. If so, its activity may be analogous to that of Abl, which possesses cytostatic activity in normal mouse fibroblasts, but not those with homozygous deficiency of pRb or p53.

CONCLUSIONS

Members of the Brk family are unusually rapidly diverging kinases, whose role appears to involve modulation of a set of pathways progressing from tyrosine kinase receptors through small GTPases and MAPK/JNK signaling to modulate a number of physiologic processes including proliferation and cell shape determination. Like Src, Abl, and other intracellular tyrosine kinases, they appear capable of activities in a range of cellular compartments including the cell membrane and the nucleus. Phenotypic effects of these proteins in vertebrates may be minor, but the two for which null mutant mice have been produced, Brk and Srm, are clearly the most closely related, and may have redundant functions. Because these kinases have not previously been viewed as a family, a variety of protein binding partners and potential signaling relationships generally have not been tested for their generality among all members. If some of these mechanisms can be shown to be conserved, it will greatly clarify the role of each of the kinases discussed.

Note added in proof: Since preparation of this review, substantial progress has been made in understanding mechanisms of Frk activity. Frk expression has been shown to block breast ductal carcinoma cell proliferation at the G_1 phase of the cell cycle (60), consistent with findings that its expression is progressively lost from human breast tumors (39). The role of Frk in inhibiting proliferation and promoting formation of cellular outgrowths in other cell lines has previously been reported by M. Welsh and colleagues, who have made extensive studies of the role of Frk in pancreatic islets which could not be properly reviewed herein. Their recent study demonstrates direct phosphorylation of the adaptor protein SHB by Frk and alteration of signaling by insulin receptor substrate proteins (61). Recently Brk was shown to negatively regulate the ability of Sam68 to increase translation of intron-containing genes containing a constitutive transport element (62), further supporting a role for Brk in posttranscriptional regulation of gene expression. Activity of overexpressed Brk in this system requires mutation of the carboxy-terminal tyrosine, which is the site of an inhibitory phosphorylation [(63); see also (36)].

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